Journal of Animal Physiology and Animal Nutrition

DOI: 10.1111/j.1439-0396.2011.01236.x

ORIGINAL ARTICLE

In vitro rumen fermentation and methane production are influenced by active components of essential oils combined with fumarate

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Keyword
essential oils, active component, monosodium fumarate, rumen fermentation, methane, microbe population

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Received: 2 December 2010;
accepted: 19 August 2011

Summary

Two trials were conducted to identify the optimal levels of essential oil active components (EOAC) and their combination with fumarate on *in vitro* rumen fermentation. In trial 1, eugenol, carvacrol, citral and cinnamaldehyde were mixed at ratios of 1:2:3:4, 2:1:4:3, 3:4:1:2, 4:3:2:1 and 1:1:1:1 to make up five combinations (EOAC1, EOAC2, EOAC3, EOAC4 and EOAC5 respectively). The mixtures were supplied at levels of 0, 50, 200 or 500 mg/l to identify the optimal combination for methane reduction. Methane production and ammonia nitrogen were decreased by adding EOAC, irrespective of component compounds, but the production of gas and total volatile fatty acids (VFA) were also decreased. Hydrogen balance analysis indicated that the ratio of hydrogen consumed via methane to hydrogen consumed via VFA was lowest at 200 mg/l of EOAC5 treatment, from which the proportional change in methane was more than the change in VFA, with 31.5% of methane reduction and 12.9% of VFA reduction. In trial 2, 200 mg/l of EOAC5 was added with 0, 5, 10 and 15 mM monosodium fumarate to see whether fumarate had a further effect on rumen fermentation. The addition of fumarate had no influence on gas production, but it further decreased methane and increased the total VFA in comparison with EOAC added solely, with the greatest decrease occurring in methane (78.1%) from 10 mM of fumarate. Quantification of the microbial populations in rumen fluids by RT-PCR showed that methanogen, protozoa, fungi, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* populations were significantly decreased by EOAC5, but were not influenced by fumarate. In summary, the addition of EOAC had consistent effects on rumen fermentation parameters, but high levels of EOAC would induce the inhibition of rumen fermentation. Adding fumarate can enhance the methane-inhibiting effect of EOAC, and the decrease was higher than that calculated stoichiometrically.

Introduction

Numerous strategies have been studied and applied to inhibit rumen methane production, including altering feed composition, improving feed quality and employing biological or chemical methane-inhibiting additives to kill off or reduce the activity of methanogens (Buddle et al., 2011; Eckard et al., 2010). The use of essential oils (EO) to inhibit methane production has been a research focus during recent years (Castillejos et al., 2006; Benchaar et al., 2008). Another methane-inhibiting strategy is the use of fumarate to scavenge hydrogen and lead to the synthesis of propionate, resulting in a decreased amount

of hydrogen available for the methanogenic pathway (Wallace et al., 2006; Ungerfeld et al., 2007).

The EOs are volatile mixtures composed of secondary metabolites of plant material, and they are characterized by diverse compositions and activities (Benchaar et al., 2008). Because of the variation in plant growing conditions, the plant parts used and the extraction methods, the EO composition of plant extracts can vary from one batch to the next (Marino et al., 2001). Thus, it is difficult to keep the compositions constant in every batch of natural EOs and to elucidate the mechanisms of EO action on rumen methane production. Active components of EO such as eugenol, thymol, cinnamaldehyde and citral have been reported to have similar effects on rumen fermentation with their correspondent natural EO, but the effects of different types of components were variable between each other (Busquet et al., 2006; Macheboeuf et al., 2008). Methane and ammonia inhibitory effects of mixtures that were composed of different types of natural EO have been evidenced by *in vitro* experiments (Newbold et al., 2004; Spanghero et al., 2008). However, little information is available on the effects of combining EO components of different chemical structures on rumen methanogenesis.

This study was conducted to investigate the effects of mixtures composed of four different EO active components (EOAC) on rumen fermentation. Additionally, it is hypothesized that the addition of fumarate can scavenge the hydrogen that accumulates in rumen fluid because of the inhibition of methanogenesis by EOAC, enabling the fibre digestion process to proceed. The first trial was to screen for the optimal combination and level of EOACs that can decrease methane production. In trial 2, fumarate was added to EOAC to investigate their combined effects on rumen fermentation. The RT-PCR method was utilized to detect the microbe community in the rumen fluids taken in trial 2.

Materials and methods

Experimental design

In trial 1, four combinations of EOAC, eugenol (2-methoxy-4-(4-propenyl)-phenol), carvacrol (2-methyl-5-isopropyl-1-phenol), citral (7-dimethyl-2, 6-octadienal) and cinnamaldehyde (3-phenyl- 2-propenal) (purity >99%; Alading Reagent Company, Shanghai, China), were mixed according to the following weight ratios to make up five different combinations: 1:2:3:4 (EOAC1), 2:1:4:3 (EOAC2), 3:4:1:2 (EOAC3), 4:3:2:1 (EOAC4) and 1:1:1:1 (EOAC5) respectively. The five combinations consisted of two aldehyde-based combinations (EOAC1 and EOAC2), two phenolic-based combinations (EOAC3 and EOAC4) and one balanced combination (EOAC5) – based on the chemical structures and characteristics of the EO components. The combinations were repeated to provide the following range of concentrations: 0, 50, 200 and 500 mg/l. An *in vitro* gas production test was conducted to identify the optimal combination and concentration to decrease ruminal methane production.

In trial 2, the optimal EOAC combination identified in trial 1 was added together with 0, 5, 10 or 15 mM monosodium fumarate (purity >99%; Yuancheng Chemical, Wuhan, China) respectively, to see whether further effects could be expected on rumen fermentation using the same *in vitro* method as in trial 1. The rumen fermentation parameters and microbial population were measured.

In vitro fermentations

In vitro fermentations were conducted in 180-ml serum bottles. The oven-dried substrate (375 mg ground corn kernels and 375 mg of ground *Leymus chinensis* hay, DM based) was weighed with four replicates into 90 ml buffer medium (Theodorou et al., 1994). Because of their insolubility, the EOAC were emulsified by Tween 80 (0.2%, v/v) before use. The concentration of Tween 80 in all of the bottles was adjusted to be the same as that in 500 mg/l EOAC-added bottles (0.002%, v/v). It has been verified that Tween 80 at such a level did not have any effect on rumen fermentation (Cong et al., 2009). Four bottles were incubated in each combination at each dose in experiments 1 and 2. Control treatments were set up to contain a substrate and a similar amount of Tween 80 but no EO and fumarate. Four bottles containing incubation medium without any substrate and additives were incubated as the blanks to correct the gas production resulting from the activity of the rumen fluid. Bottles with substrate, additives and incubation medium were stored at 39 °C overnight after sealing with a butyl rubber stopper and aluminium caps.

In the morning of the second day, rumen contents were obtained before the morning feeding equally from three rumen-fistulated Hu sheep fed on a maintenance diet (850 g/day, as fed) that consisted of *L. chinensis* (Trin.) hay (450 g/day) and a concentrate mixture (400 g/day). The ingredients of the concentrate included corn, soybean cake and wheat bran. The composition (% of DM) of the diet was CP

10.9, NDF 42.6 and ADF 26.4. Sheep were fed twice daily at 8:30 and 17:00 h. The rumen contents obtained were mixed and strained through four layers of cheesecloth into a flask under CO_2 in the water bath at 39 °C until used. Filtered rumen fluid (10 ml) was injected through the stopper using a syringe into the incubation bottles, and then the bottles were shaken to mix the contents and were placed in an incubator at 39 °C. The pressure and methane production at 24 h were measured according to methods described by Zhang et al. (2008). At the end of the incubation, the incubation fluids were sampled and samples were stored at −20 °C for an analysis of the end products. In trial 2, subsamples of incubation fluid were also taken and stored at −80 °C for the analysis of the microbe communities.

Chemical analytical procedures

Methane production in the headspace gas after 24 h was analysed by gas chromatography (GC-2010; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a capillary column (HP-INNOWAX, 1909N-133), as described by Zhang et al. (2008). Incubation fluids were collected into tubes for the analysis of pH, ammonia nitrogen (N) and volatile fatty acids (VFA). The concentration of ammonia-N was analysed by colorimetry (Broderick and Kang, 1980). Samples for VFA analysis were prepared and analysed by gas chromatography (Jouany, 1982). The final pH was determined at the end of the incubation using a pH meter (model PB-10/C; Sartorius, Göettingen, Germany).

Analysis of rumen microbe population

The DNA of rumen microbes present in incubations in trial 2 was extracted by the bead-beating method with the mini-bead beater (Biospec Products, Bartlesville, OK, USA), as described by Zhang et al. (2008). The PCR primers designed for total bacteria, fungi, protozoa, methanogens and two cellulolytic bacteria (*Fibrobacter succinogenes* and *Ruminococcus flavefaciens*) were cited from Denman and McSweeney (2006) and Denman et al. (2007). Quantitative PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex Taq II Perfect Real Time (TaKaRa Bio, Dalian, China). The PCR mixture consisted of 2 μl template DNA, 0.2 mM dNTP, 0.3 μM Primer, 1.5 mM $MgCl_2$ and 1.25 U Taq in total 20-μl volume. The amplification procedure involved denaturation at 95 °C for 10 s, then 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Melting curve analysis was performed after amplification to verify the specificity of the real-time PCR. The amplification efficiencies for each primer pair were investigated by examining the dilution series of the total rumen microbial DNA template on the same plate in triplicate.

Data calculation and statistical analysis

Hydrogen recovery (%) was estimated as $(4M + 2P + 2B)/(2A + P + 4B) \times 100$, and the ratio of hydrogen consumed via CH4/VFA was estimated as $4M/(2P + 2B)$, where acetate (A), propionate (P), butyrate (B) and methane (M) production were expressed in mmol (Demeyer, 1991). The reduction in methane production by additives was usually accompanied by a reduction in VFA production. The reductions in the methane and VFA production were expressed as a proportion of the total production in the controls and were calculated using the following equation:

$$1 - \frac{\text{Methane or VFA production in treated incubations}}{\text{Methane or VFA production in incubation with no added control}}$$

The relative methane reduction potential (RMRP) of a treatment versus control was calculated as a ratio of the reduction in methane production to the reduction in VFA production and was used to screen for the optimal combination.

Quantification for methanogen, protozoa, fungi, *R. flavefaciens* and *F. succinogenes* was expressed as a proportion of the total rumen bacterial 16S rDNA according to the following equation: Relative quantification (% of total bacterial 16S rDNA) = $2^{-(C_{t\,\text{target}} - C_{t\,\text{totalbacteria}})} \times 100$, where C_t represents threshold cycle.

In vitro incubation data of trial 1 were analysed as a factorial design (5 EOAC × 4 levels) using the PROC GLM procedure of SAS (SAS Institute, 2005), according to the following statistical model: $Yij = \mu + \alpha i + \beta\varpi j + (\alpha \times \beta)\, ij + \varepsilon ij$, in which Yijk is the dependent variable, μ is the overall mean, αi is the effect of the EOACs (i = 1, 5), βj is the effect of the level (j = 1, 4) and εij is the residual error. The data in trial 2 were analysed with a one-way analysis of variance using the PROC GLM procedure of SAS (SAS Institute, 2005).

Table 1 Effect of different combinations of active components from essential oils on 24 h of *in vitro* rumen fermentation (parameters and hydrogen balance)

		Combined components*						p Value		
Items	Level (mg/l)	EOAC1	EOAC2	EOAC3	EOAC4	EOAC5	SEM	EOAC	Dose	Int.
Final pH	0	6.85	6.85	6.88	6.91	6.87	0.032	0.026	0.163	0.007
	50	6.87^{ab}	6.84^{b}	6.88^{ab}	6.91^{a}	6.87^{ab}				
	200	6.85^{b}	6.82^{b}	6.86^{ab}	6.92^{a}	6.86^{ab}				
	500	6.83^{b}	6.81^{b}	6.85^{ab}	6.90^{a}	6.90^{a}				
Gas produced (ml)	0	91.6	91.9	91.4	91.6	91.4	1.75	0.012	0.005	0.016
	50	86.8	87.8	86.2	85.1	86.5				
	200	74.3^{b}	75.2^{ab}	77.8^{a}	76.9^{ab}	76.9^{ab}				
	500	63.6^{b}	64.4^{b}	67.9^{a}	67.4^{a}	68.5^{a}				
Methane produced	0	0.94	0.96	0.95	0.96	0.96	0.03	0.007	<0.001	0.153
(mmol)	50	0.95^{a}	0.94^{a}	0.88^{b}	0.94^{a}	0.85^{b}				
	200	0.68	0.70	0.70	0.67	0.66				
	500	0.32^{bc}	0.43^{a}	0.37^{b}	0.34^{b}	0.28^{c}				
Final NH_3-N concentration	0	11.6	11.6	11.7	11.7	11.6	0.223	0.015	0.004	0.061
(mg N/dl)	50	10.7^{a}	10.6^{a}	9.9^{b}	10.4^{a}	10.8^{a}				
	200	8.8^{b}	9.2^{b}	10.3^{a}	10.3^{a}	9.1^{b}				
	500	10.5^{b}	10.5^{b}	11.1^{b}	10.3^{a}	9.5^{c}				
TVFA (mmol)	0	3.73	3.73	3.72	3.72	3.72	0.052	<0.001	<0.001	0.003
	50	3.56^{b}	3.54^{b}	3.68^{a}	3.68^{a}	3.62^{ab}				
	200	3.09^{b}	3.08^{b}	3.24^{a}	3.19^{ab}	3.24^{a}				
	500	1.40^{d}	1.45^{d}	1.58^{bc}	1.49^{dc}	1.65^{ab}				
Acetate/propionate	0	2.18	2.16	2.25	2.23	2.24	0.096	0.032	<0.001	0.001
	50	1.96^{b}	2.07^{ab}	2.04^{ab}	2.20^{a}	1.96^{b}				
	200	1.87^{b}	2.08^{a}	1.95^{ab}	1.97^{ab}	1.81^{b}				
	500	4.98^{d}	6.00^{a}	5.56^{b}	5.39^{c}	5.97^{a}				
H recovery (%)	0	87.4	87.9	87.5	87.9	87.8	1.72	0.026	<0.001	0.289
	50	92.2	91.1	88.5	88.8	89.3				
	200	87.0	88.6	86.0	86.3	85.2				
	500	84.1^{a}	81.8^{a}	78.2^{ab}	78.8^{ab}	76.7^{b}				
2H consumed via	0	1.45	1.46	1.45	1.44	1.45	0.051	<0.001	<0.001	0.025
CH_4/via VFA	50	1.32^{b}	1.32^{b}	1.43^{a}	1.44^{a}	1.41^{b}				
	200	1.26	1.22	1.16	1.14	1.21				
	500	0.98^{a}	0.94^{a}	0.78^{b}	0.75^{b}	0.42^{c}				

NH_3-N, ammonia-N; TVFA, total volatile fatty acid.

*EOAC1, EOAC2, EOAC3, EOAC4, EOAC5: combination of eugenol, carvacrol, citral and cinnamaldehyde at ratios of 1:2:3:4, 2:1:4:3, 3:4:1:2, 4:3:2:1 and 1:1:1:1 respectively.

Means within a row with different superscripts differ ($p < 0.05$).

Results

Effects of EOAC on *in vitro* rumen fermentation parameters

A dose of EOAC did not significantly influence pH value, but a combination has a significant influence on the pH (Table 1). Gas production was decreased with an increasing level of EOAC combinations ($p < 0.01$), and the type of EOAC has a significant effect on gas production ($p = 0.012$). Methane production was decreased in an EOAC-dose-dependent manner, but the inhibition of methane production was much greater than the inhibition of gas and total VFA. The type of EOAC combinations influenced the methane production ($p < 0.01$). The final ammonia-N concentration tended to be decreased by EOAC, with most EOAC combinations showing the least ammonia-N at the 200 mg/l level. The final total VFA production decreased with an increasing level of combined EOAC, and the greatest decrease (more than 50%) occurred in 500 mg/l of EOAC in all of the groups. An interaction between the EOAC types and level was observed in the pH, gas, total VFA and A/P ratio ($p < 0.05$). The aldehyde-based combinations (EOAC1 and 2) showed a higher inhibitory effect on the total VFA than the phenolic-based and balanced combinations (EOAC 3, 4 and 5) at every EOAC level.

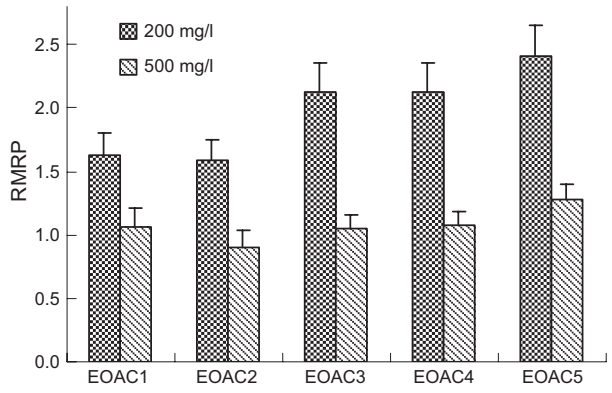

Fig. 1 Relative methane reduction potential (RMRP) when different combinations of essential oil active components (EOAC) were added at 200 or 500 mg/l. RMRP = Ratio of methane production reduction relative to reduced total volatile fatty acids production because of the addition of different EOAC combinations at varying levels; EOAC1, EOAC2, EOAC3, EOAC4 and EOAC5 = combination of eugenol, carvacrol, citral and cinnamaldehyde at ratios of 1:2:3:4, 2:1:4:3, 3:4:1:2, 4:3:2:1 and 1:1:1:1 on weight basis respectively. Addition of EOAC5 at 200 mg/l had the highest RMRP and was screened as the optimal combination. Bar = standard error of mean.

The type and dose of EOAC were significantly influenced by the hydrogen recovery and the ratio of hydrogen consumed via methane to hydrogen consumed via VFA production. At the level of 50 mg/l, the hydrogen recovery was higher in the two aldehyde-based combinations (EOAC1 and EOAC2) than in the other groups ($p < 0.05$). The hydrogen recovery of all of the EOAC combinations at the level of 500 mg/l was lower than at the levels of 50 and 200 mg/l, while no significant difference was observed in the hydrogen recovery when 200 mg/l of EOAC was added. The hydrogen that was consumed via methane was greater than that via VFA, with a higher ratio in the two aldehyde-based combinations (EOAC1 and EOAC2) than in other combinations at each EOAC level. The ratio was lower in the groups with 200 mg/l of added EOAC compared with other additional levels, with the lowest ratio occurring in the group with 200 mg/l of added EOAC5. As can be seen from Fig. 1, the RMRP was higher in the 200 mg/l of EOAC than that in 500 mg/l-added group, while at the 200 mg/l EOAC level, the RMRP value of the phenolic-based and balanced combinations (EOAC 3, 4 and 5) were significantly higher than that of the aldehyde-based combinations (EOAC1 and 2). The highest RMRP was at 200 mg/l of EOAC5, in which methane was decreased by 31.5%, while the total VFA decreased by 12.9%. Taking methane and VFA production together into account, this level of EOAC5 could be screened as the optimal combination for methane production inhibition.

Effect of fumarate with EOAC5 on rumen fermentation parameters

No difference was observed in the final pH value within each group (Table 2). Gas production was lower in the four EOAC treatments with or without fumarate in relation to the controls ($p < 0.05$), but no difference was observed among the different levels of fumarate. Methane production was further inhibited by fumarate, compared with EOAC added solely ($p < 0.05$), with the greatest inhibition (78.1%) at 10 mM of fumarate. Ammonia-N was significantly decreased in the EOAC5-added groups, compared with the control. The addition of fumarate at 5 or 10 mM increased the total VFA compared with the EO added solely ($p < 0.05$), but the production was still lower than that in the control. The acetate proportion was significantly decreased in EOAC5 without fumarate compared with the control ($p < 0.05$), but no difference was observed among the other groups, while the propionate proportion was increased by the inclusion of EOAC ($p < 0.05$), with no significant difference among the different levels of fumarate.

The ratio of H consumed via methane production to hydrogen consumed via VFA production was significantly decreased by the addition of fumarate ($p < 0.01$), but H recovery was also decreased ($p < 0.05$, Table 2), indicating that the production was not completely redirected to VFA production. The RMRP in fumarate-added treatments was higher than in the group without fumarate ($p < 0.05$), with the highest RMRP at 10 mM of fumarate.

Effect of fumarate along with EOAC on the ruminal microbe population

Populations of several fluid-associated microbes relative to the total bacterial 16S rDNA (%) are shown in Table 3. The methanogen population was significantly decreased in 200 mg/l EOAC5-added groups with or without fumarate ($p < 0.05$), and the addition of fumarate at 10 mM resulted in the lowest methanogen populations. The protozoa population was sharply decreased in all of the EOAC5-added groups (>85%), while protozoa in 15 mM fumarate was greater than in the 0, 5 and 10 mM groups ($p < 0.05$). Similar to protozoa, the population of fungi and two cellulolytic bacteria, *F. succinogenes* and *R. flavefaciens,* were significantly inhibited by

Table 2 Effect of combined active components from essential oils* (200 mg/l) with different doses of monosodium fumarate on 24 h of *in vitro* rumen fermentation parameters

		Fumarate level (mM)†				
Parameters	Control	0	5	10	15	SEM
Final pH	6.35	6.33	6.25	6.33	6.32	0.04
Gas produced (ml)	88.2^{a}	76.2^{b}	75.4^{b}	74.9^{b}	73.1^{b}	1.65
Methane produced (mmol)	0.93^{a}	0.64^{b}	0.22^{c}	0.14^{d}	0.32^{c}	0.034
Final NH_3-N concentration (mg N/dl)	12.5^{a}	10.3^{b}	9.8^{b}	8.9^{c}	10.0^{b}	0.32
Total VFA (mmol)	3.47^{a}	3.00^{c}	3.13^{b}	3.15^{b}	3.08^{bc}	0.072
Molar proportion (%)						
Acetate	61.1^{a}	58.2^{b}	60.1^{ab}	58.6^{ab}	60.3^{ab}	1.42
Propionate	27.3^{a}	32.7^{b}	30.6^{b}	31.5^{b}	30.6^{b}	1.25
Butyrate	11.0^{a}	9.1	9.3	9.9	9.1	0.71
Acetate/propionate	2.33^{a}	1.79^{c}	2.01^{b}	1.88^{bc}	2.03^{a}	0.052
2H generated (mmol)	7.11^{a}	6.07^{b}	6.24^{b}	6.17^{b}	6.01^{b}	0.164
2H consumed (mmol)	6.24^{a}	5.05^{b}	3.59^{c}	3.20^{d}	3.63^{c}	0.159
via CH_4/via VFA	1.32^{a}	0.89^{b}	0.43^{c}	0.22^{d}	0.39^{c}	0.084
H recovery rate (%)	86.5^{a}	84.2^{a}	58.1^{b}	52.5^{c}	61.3^{b}	2.78
RMRP	_	2.25^{c}	6.11^{b}	7.47^{a}	5.21^{b}	0.403

EOAC, essential oil active components; NH_3-N, ammonia-N; VFA, volatile fatty acid; RMRP, relative methane reduction potential of a treatment versus control, which was calculated as a ratio of reduction in methane production to reduction in VFA production.
Means within a row with different superscripts differ ($p < 0.05$).
*A mixture of eugenol, carvacrol, citral and cinnamaldehyde at an equal ratio.
†EOAC at 200 mg/l final concentration was present in all of these incubations, which were conducted in triplicate.

Table 3. Effect of combined active components from essential oils* (200 mg/l) with different doses of monosodium fumarate on rumen microbe populations (% of total bacterial 16S rDNA)

		Fumarate level (mM)†				
Microbes	Control	0	5	10	15	SEM
Methanogen	0.55^{a}	0.42^{b}	0.46^{b}	0.36^{c}	0.46^{b}	0.036
Protozoa	2.68^{a}	0.31^{bc}	0.38^{bc}	0.30^{b}	0.47^{c}	0.064
Fungi ($\times 10^{-4}$)	83.36^{a}	0.12^{b}	0.36^{b}	0.57^{b}	0.49^{b}	9.125
Fibrobacter succinogenes ($\times 10^{-3}$)	4.33^{a}	0.06^{b}	0.12^{b}	0.14^{b}	0.31^{b}	0.512
Ruminococcus flavefaciens ($\times 10^{-5}$)	5.84^{a}	0.02^{b}	0.04^{b}	0.02^{b}	0.06^{b}	0.602

EOAC, essential oil active components.
Means within a row with different superscripts differ ($p < 0.05$).
*A mixture of eugenol, carvacrol, citral and cinnamaldehyde at an equal ratio.
†EOAC at 200 mg/l final concentration was present in all of these incubations, which were conducted in triplicate.

200 mg/l of EOAC5. The addition of fumarate tended to increase the fungi and *R. flavefaciens* populations ($p > 0.05$) compared with EOAC5 added solely.

Discussion

Essential oils are complex chemical mixtures composed of constituents, and their biological activities may be achieved by synergistic effects of their different components (Chaieb et al., 2007; Bakkali et al., 2008). A combination of different types of EO components that exert their activities through varying mechanisms together would lead to the production of new EOs with different bioactivity (Newbold et al., 2004; Spanghero et al., 2008). In this study, although aldehyde-based combinations (EOAC1 and EOAC2) showed influence on methane production similar to phenolic-based combinations (EOAC3 and EOAC4), their inhibitory effects on the total VFA was greater than those exerted by the phenolic-based combinations at every EOAC level, which resulted in lower RMRP in EOAC1 and EOAC2 at 200 mg/l level. A balanced combination (EOAC5)

had a greater methane reduction ability than other combinations, indicating that a blending of EO components with different action modes together would be a potential way to inhibit ruminal methane, which was consistent with other research (Newbold et al., 2004; Cardozo et al., 2006).

In most cases, the use of EO to manipulate rumen methane production *in vitro* was accompanied by the inhibition of fermentation (Benchaar et al., 2008). To control the inhibitory effects on rumen fermentation, the additional level should be considered. In the current study, the level of EOAC had a significant effect on the gas production, methane, ammonia-N and total VFA. In addition, EOAC types and levels have an interaction effect on the pH, gas, total VFA and A/P ratio, indicating that the effects of EOAC on these parameters varied with their additional level. As a result, a certain level should be identified when we investigate the effect of EOAC on rumen fermentation. In an *in vitro* experiment, Calsamiglia et al. (2007) found that the suitable level of EO was approximately 500 mg/l. However, in the current study, 500 mg/l of EOAC sharply reduced the total VFA production, while 50 mg/l of EOAC had only a small influence on the methane production, indicating that the high or the low level was not suitable for manipulating the rumen methane production. The ratio of hydrogen that is consumed via methane to hydrogen that is consumed via VFA was lower (Table 1), while the RMRP was higher at a level of 200 mg/l than at 500 mg/l (Fig. 1), with the highest RMRP occurring in the 200 mg/l EOAC5 treatment. From the aforementioned results, it is inferred that the optimal combination for methane production inhibition is when a mixture of eugenol, carvacrol, citral and cinnamaldehyde at an equal weight ratio is added at 200 mg/l.

Methanogenesis is the main way to consume hydrogen produced by fibre digestion, which can make rumen fermentation proceed in a positive way (Mohammed et al., 2004). The inhibition of methanogenesis by EOAC could induce hydrogen accumulation or hydrogen could be consumed by the production of VFA. However, this scenario is not always the case. Not all of the hydrogen can be consumed by the rumen bacteria to produce VFA. Fumarate has been extensively researched as an alternative H_2 sink (López et al., 1999; Ungerfeld et al., 2007; Wood et al., 2009). Our results showed that the addition of different levels of fumarate further decreased methane production in relation to EOAC5, with the greatest decrease (78.1%) occurring at 10 mM of fumarate. The addition of fumarate at 5 or 10 mM increased the total VFA production and decreased the ratio of the hydrogen that was consumed via CH_4/via VFA, suggesting that fumarate would be converted to VFA by scavenging the hydrogen (Table 3). However, H recovery in the fumarate-added groups was still lower than the control or solely EOAC-added group. This is observed most likely because the methanogenesis was further inhibited by fumarate, while fumarate could not sink all of the surplus hydrogen.

In terms of stoichiometric calculations, 0.25 mmol of methane can be avoided by the production of 1 mmol of fumarate (Ungerfeld et al., 2007). In the current study, methane was decreased from 0.64 mmol in the EOAC treatment without fumarate to 0.14 mmol in 10 mM of fumarate-added treatment, indicating a 0.50-mmol methane reduction by 1 mmol of fumarate addition (10 mM × 0.1 l incubation fluid). The result was consistent with Wood et al. (2009), who observed that methane was decreased much more than is predicted stoichiometrically when fumarate was added in an *in vivo* experiment. In this study, the greater decrease in methane than the expected amount stoichiometrically should not be attributed mainly to fumarate, but instead it should be attributed to the combined effect of EOAC and fumarate. The reason for the further reduction in methane when fumarate was added in combination with EOAC is that the activity or community of methane-producing microbes was inhibited by fumarate, because the population of methanogens was not influenced by fumarate (Table 3). A similar phenomenon can also be found in studies on other methane inhibitors, such as tea saponin (Guo et al., 2008), vaccine (Williams et al., 2009) or unsaturated long-chain fatty acids (Zhang et al., 2008).

Selective inhibition by EO of specific microbes, such as methanogens, protozoa and hyper-ammonia-producing bacteria, has been reported and was regarded as the main mechanism of EOs for manipulating rumen fermentation (McIntosh et al., 2003; Calsamiglia et al., 2007). Methanogen and protozoa populations were reduced by EOAC5, but fungi and cellulolytic bacteria, the two important fibre-digesting groups, were also decreased by EOAC5. The overall inhibition of microbes by EOAC indicated that fibre digestion has been influenced. This influence could be a reason for the reduction in the total VFA in the EOAC-added groups. In general, defaunation, anti-fungi and methanogen-inhibiting effects are the main mechanisms with which the EOAC

combinations exert their effect on *in vitro* fermentation. The addition of an EOAC combination with fumarate is effective in decreasing ruminal methane production *in vitro;* thus, further alternative hydrogen sink pathways should be considered.

Conclusions

A combination of different EOACs that have a variety of action modes would be a promising measure to inhibit methane production. The effects of EOs on rumen fermentation parameters were consistent, irrespective of the component compounds, but a high level of EOAC would induce the inhibition of rumen fermentation. A coupled addition of fumarate with EOAC can further decrease methane production compared with EOAC added solely, causing greater suppression of methanogen than that calculated stoichiometrically. The addition of fumarate alleviated the VFA-decreasing effect caused by EOACs, but it could not alleviate the inhibition of fungi and cellulolytic bacteria by EOAC. Further study is needed to investigate why the inhibition of methanogenesis is greater than that predicted stoichiometrically for the addition of EOAC and fumarate together.

Acknowledgments

This work was supported partly by grants from the National Natural Science Foundation of China (No.30972105) and China–Australia Special Fund for Science and Technology (No. 2010DFA31040). The authors gratefully acknowledge Ms. S.P. Doto for her help in English writing.

Reference

Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M., 2008: Biological effects of essential oils – a review. *Food and Chemical Toxicology* **46**, 446–475.

Benchaar, C.; Calsamiglia, S.; Chaves, A. V.; Fraser, G. R.; Colombatto, D.; McAllister, T. A.; Beauchemin, K. A., 2008: A review of plant-derived essential oils in ruminant nutrition and production. *Animal Feed Science and Technology* **145**, 209–228.

Broderick, G. A.; Kang, J. H., 1980: Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. *Journal of Dairy Science.* **63**, 64–75.

Buddle, B. M.; Denis, M.; Attwood, G. T.; Altermann, E.; Janssen, P. H.; Ronimus, R. S.; Pinares-Patiño, C. S.; Muetzel, S.; Neil Wedlock, D., 2011: Strategies to reduce methane emissions from farmed ruminants grazing on pasture. *The Veterinary Journal* **188**, 11–17.

Busquet, M.; Calsamiglia, S.; Ferret, A.; Kamel, C., 2006: Plant extracts affect in vitro rumen microbial fermentation. *Journal of Dairy Science* **89**, 761–771.

Calsamiglia, S.; Busquet, M.; Cardozo, P. W.; Castillejos, L.; Ferret, A., 2007: Invited review: essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science* **90**, 2580–2595.

Cardozo, P. W.; Calsamiglia, S.; Ferret, A.; Kamel, C., 2006: Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high-concentrate diet. *Journal of Dairy Science* **84**, 2801–2808.

Castillejos, L.; Calsamiglia, S.; Ferret, A., 2006: Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. *Journal of Dairy Science* **89**, 2649–2658.

Chaieb, K.; Hajlaoui, H.; Zmantar, T.; Kahla- Nakbi, A. B.; Rouabhia, M.; Mahdouani, K.; Bakhrouf, A., 2007: The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum L.* Myrtaceae): a short review. *Phytotherapy Research* **21**, 501–506.

Cong, Z. H.; Tang, S. X.; Tan, Z. L.; Sun, Z. H.; Zhou, C. S.; Han, X. F.; Wang, M.; Ren, G. P., 2009: Effects of different nonionic surfactants on in vitro fermentation characteristics of cereal straws. *Journal of Animal Science* **87**, 1085–1096.

Demeyer, D. I., 1991: Quantitative aspects of microbial metabolism in the rumen and hindgut. In: J. P. Jouany (ed.), *Rumen Microbial Metabolism and Ruminant Digestion*. INRA Editions, Paris, France, pp. 217–237.

Denman, S. E.; McSweeney, C. S., 2006: Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiology Ecology.* **58**, 572–582.

Denman, S. E.; Tomkins, N. W.; McSweeney, C. S., 2007: Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiology Ecology.* **62**, 313–322.

Eckard, R. J.; Grainger, C.; de Klein, C. A. M., 2010: Options for the abatement of methane and nitrous oxide from ruminant production: a review. *Livestock Science* **130**, 47–56.

Guo, Y. Q.; Liu, J.-X.; Lu, Y.; Zhu, W. Y.; Denman, S. E.; McSweeney, C. S., 2008: Effect of tea saponin on methanogenesis, microbial community structure and expression of *mcrA* gene, in cultures of rumen microorganisms. *Letters in Applied Microbiology* **47**, 421–426.

Jouany, J. P., 1982: Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial

cultures and anaerobic fermenter contents. *Sciences des Aliments* **2**, 131–144.

López, S.; Valdés, C.; Newbold, C. J.; Wallace, R. J., 1999: Influence of sodium fumarate addition on rumen fermentation in vitro. *British Journal of Nutrition* **81**, 59–64.

Macheboeuf, D.; Morgavi, D. P.; Papon, Y.; Mousset, J. L.; Arturo-Schaan, M., 2008: Dose-response effects of essential oils on in vitro fermentation activity of the rumen microbial population. *Animal Feed Science and Technology* **145**, 335–350.

Marino, M.; Bersani, C.; Comi, G., 2001: Impedance measurements to study the antimicrobial activity of essential oils from Lamiacea and Compositae. *International Journal of Food Microbiology* **67**, 187–195.

McIntosh, F. M.; Williams, P.; Losa, R.; Wallace, R. J.; Beever, D. A.; Newbold, C. J., 2003: Effects of essential oils on ruminal microorganisms and their protein metabolism. *Applied and Environmental Microbiology* **69**, 5011–5014.

Mohammed, N.; Lila, Z. A.; Ajisaka, N.; Hara, K.; Mikuni, K.; Kanda, S.; Itabashi, H., 2004: Inhibition of ruminal microbial methane production by β-cyclodextrin iodopropane, malate and their combination in vitro. *Journal of Animal Physiology and Animal Nutrition* **88**, 188–195.

Newbold, C. J.; McIntosh, F. M.; Williams, P.; Losa, R.; Wallace, R. J., 2004: Effects of a specific blend of essential oil compounds on rumen fermentation. *Animal Feed Science and Technology* **114**, 105–112.

SAS Institute, 2005: *SAS Online Doc version 9.1.3*. SAS Inst., Cary, NC.

Spanghero, M.; Zanfi, C.; Fabbro, E.; Scicutella, N.; Camellini, C., 2008: Effects of a blend of essential oils on some end products of in vitro rumen fermentation. *Animal Feed Science and Technology* **145**, 364–374.

Theodorou, M. K.; Williams, B. A.; Dhanoa, M. S.; McAllan, A. B.; France, J., 1994: A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feed. *Animal Feed Science and Technology* **48**, 185–197.

Ungerfeld, E. M.; Kohn, R. A.; Wallace, R. J.; Newbold, C. J., 2007: A meta-analysis of fumarate effects on methane production in ruminal batch cultures. *Journal of Animal Science* **85**, 2556–2563.

Wallace, R. J.; Wood, T. A.; Rowe, A.; Price, J.; Yanez, D. R.; Williams, S. P.; Newbold, C. J., 2006: Encapsulated fumaric acid as a means of decreasing ruminal methane emissions. *International Congress Series* **1293**, 148–151.

Williams, Y. J.; Popovski, S.; Rea, S. M.; Skillman, L. C.; Toovey, A. F.; Northwood, K. S.; Wright, A.-D. G., 2009: A vaccine against rumen methanogens can alter the composition of archaeal populations. *Applied and Environmental Microbiology* **75**, 1860–1866.

Wood, T. A.; Wallace, R. J.; Rowe, A.; Price, J.; Yáñez-Ruiz, D. R.; Murray, P.; Newbold, C. J., 2009: Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. *Animal Feed Science and Technology* **152**, 62–71.

Zhang, C. M.; Guo, Y. Q.; Yuan, Z. P.; Wu, Y. M.; Wang, J. K.; Liu, J. X.; Zhu, W. Y., 2008: Effect of octadeca carbon fatty acids on microbial fermentation, methanogenesis and microbial flora in vitro. *Animal Feed Science and Technology* **146**, 259–269.